

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 5 :</b> <b>A61K 48/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 93/04701</b> <b>(43) International Publication Date:</b> 18 March 1993 (18.03.93)
<b>(21) International Application Number:</b> PCT/US92/07339 <b>(22) International Filing Date:</b> 4 September 1992 (04.09.92)  <b>(30) Priority data:</b> 07/755,083 5 September 1991 (05.09.91) US 07/788,119 4 November 1991 (04.11.91) US 07/864,003 3 April 1992 (03.04.92) US  <b>(71) Applicant:</b> UNIVERSITY OF CONNECTICUT [US/US]; Storrs, CT 06269 (US). <b>(72) Inventors:</b> WU, George, Y. ; WU, Catherine, H. ; 15 Rundelane, Bloomfield, CT 06002 (US). <b>(74) Agents:</b> DeCONTI, Giulio, A., Jr. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).		<b>(81) Designated States:</b> AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> TARGETED DELIVERY OF POLY- OR OLIGONUCLEOTIDES TO CELLS  <b>(57) Abstract</b>  Molecular complexes for targeting oligonucleotides, such as antisense oligonucleotides or ribozymes, to a specific cell to block expression of a gene or genes in the cell are described. The single-stranded poly- or oligonucleotide is complexed to a conjugate of a cell-specific binding agent and a poly- or oligonucleotide-binding agent. The cell-specific binding agent is specific for a cellular surface structure which mediates internalization of the complex. An example is the asialoglycoprotein receptor of hepatocytes. The poly- or oligodeoxy-nucleotide-binding agent is a compound such as a polycationic protein which stably complexes the oligonucleotide under extracellular conditions and releases it under intracellular conditions so that it can hybridize with the target RNA. The molecular complex is stable and soluble in physiological fluids and can be used to selectively introduce antisense oligonucleotides, ribozymes or other signal-stranded oligonucleotides into a cell to inhibit expression of a gene within the cell. The oligonucleotide can be directed against cellular genes (e.g., cellular oncogenes) or genes of noncellular origin (e.g., viral oncogenes, genes of an infecting pathogen).		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MN	Mongolia
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Faso	GN	Guinea	NO	Norway
BG	Bulgaria	GR	Greece	NZ	New Zealand
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	PT	Portugal
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovak Republic
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CS	Czechoslovakia	LU	Luxembourg	SU	Soviet Union
CZ	Czech Republic	MC	Monaco	TD	Chad
DE	Germany	MG	Madagascar	TG	Togo
DK	Denmark	ML	Mali	UA	Ukraine
ES	Spain			US	United States of America

-1-

TARGETED DELIVERY OF POLY- OR  
OLIGONUCLEOTIDES TO CELLS

Background of the Invention

Antisense oligonucleotides hold great promise as means of specifically inhibiting unwanted gene expression in cells. Improvements in the delivery of  
05 oligonucleotides to cells will enhance effectiveness. Naked antisense oligonucleotides can be taken up by cells non-specifically and at low efficiency. Some methods have been explored to increase uptake. Lemaitre et al. covalently coupled  
10 an oligonucleotide to polylysine and demonstrated inhibition of viral gene expression at several fold lower than DNA concentrations compared to mixtures of polylysine and antisense DNA (Lemaitre, M. et al. Proc. Natl. Acad. Sci. USA 84:648-652). Although  
15 specific antiviral effects were shown, specific delivery was not demonstrated.

Summary of the Invention

This invention pertains to a soluble, targetable molecular complex for targeting poly- or oligonucleotides to a specific cell to inhibit the expression of  
05 a gene or genes. The molecular complex comprises a single-stranded poly- or oligonucleotide which hybridizes to an RNA transcript of the gene, complexed to a carrier which is a conjugate of a cell-specific binding agent and a poly- or  
10 oligonucleotide-binding agent. The complex is administered in a pharmaceutically acceptable solution in an amount sufficient to hybridize to and inhibit the function of the RNA transcript.

The poly- or oligonucleotide can be DNA or RNA.  
15 For antisense applications, an antisense oligodeoxynucleotide can be used which hybridizes to and inhibits the function of an RNA. The targeted RNA is typically a messenger RNA. The oligonucleotide can also be an RNA which has catalytic activity (a  
20 ribozyme). The target for antisense or ribozyme-mediated inhibition can be a gene or genes of cellular origin (e.g., a cellular oncogene) or of noncellular origin (e.g., a viral oncogene or the genes of an infecting pathogen such as a virus).

The cell-specific binding agent is specific for a cellular surface structure, typically a receptor, which mediates internalization of bound ligands by endocytosis, such as the asialoglycoprotein receptor of hepatocytes. The cell-specific binding agent can be a natural or synthetic ligand (for example, a protein, polypeptide, glycoprotein, carbohydrate, etc.) or it can be an antibody, or an analogue thereof, which specifically binds a cellular surface structure which then mediates internalization of the bound complex. The poly- or oligonucleotide-binding component of the conjugate is a compound such as a polycation which stably complexes the single-stranded poly- or oligonucleotide under extracellular conditions and releases it under intracellular conditions so that it can function within the cell.

The complex of the gene and the carrier can be used in vitro or in vivo to selectively deliver poly- or oligonucleotides to target cells. The complex is stable and soluble in physiological fluids. It can be administered in vivo where it is selectively taken up by the target cell via the surface-structure-mediated endocytotic pathway. The incorporated poly- or oligonucleotide hybridizes with its complementary RNA, thereby inhibiting function of the RNA and expression of the target gene or genes.

#### Brief Description of the Figures

Figure 1 shows the uptake of complexed antisense DNA by HepG2 and SK Hep1 cells.

Figure 2 shows the effect of complexed antisense DNA on hepatitis B virus surface antigen concentration in culture medium.

Figure 3 shows Southern blots of DNA extracted from medium and cells after 24 hrs of exposure to antisense DNA.

Detailed Description of the Invention

A soluble, targetable molecular complex is used to selectively deliver a single-stranded poly- or oligonucleotide to a target cell or tissue in vivo to specifically inhibit gene expression. The molecular complex comprises the oligonucleotide to be delivered complexed to a carrier made up of a binding agent specific for the target cell and a DNA-binding agent. The complex is selectively taken up by the target cell and the oligonucleotide hybridizes to the RNA transcript which inhibits expression of the targeted gene(s).

The poly- or oligonucleotide is a single-stranded molecule which hybridizes to a specific RNA under intracellular conditions. The degree of complementarity required for appropriately specific hybridization to the target RNA sequence under intracellular conditions can be determined empirically.

In a preferred embodiment, the oligonucleotide is an antisense oligodeoxynucleotide. The antisense oligodeoxynucleotide can be a normal oligodeoxynucleotide or an analogue of an oligodeoxynucleotide (e.g., phosphorothioate oligonucleotides, in which one of the phosphate oxygens is replaced by a sulfur atom) sufficiently stable to reach the target in effective concentrations. See e.g., Stein, C.A. and Cohen, J.S. (1988) Cancer Research 48:2659-2668. Antisense oligodeoxynucleotides can be prepared by standard synthetic procedures.

The antisense oligonucleotides can be designed to operate by different mechanisms of gene inhibition. Generally, these mechanisms involve the hybridization of the oligonucleotide to a specific

RNA sequence, typically a messenger RNA. The targeted sequence can be located in the coding region of the RNA or it can be a signal sequence required for processing or translation of the RNA. The  
05 targeted sequence can be a sequence normally found in an organism or a sequence found in a pathogenic organism but not in its host. Alternatively, the oligonucleotide may form a triple helix DNA structure, inhibiting transcription of the mRNA  
10 sequence.

In other embodiments, the oligonucleotide can be an RNA molecule which has catalytic activity, i.e., a ribozyme. Ribozymes are advantageous because they specifically cleave and, thus, destroy the targeted  
15 RNA sequence. Ribozymes are described in U.S. Patent No. 4,987,071.

The carrier component of the complex is a conjugate of a cell-specific binding agent and an oligonucleotide-binding agent. The cell-specific  
20 binding agent specifically binds a cellular surface structure which mediates its internalization by, for example, the process of endocytosis. The surface structure can be a protein, polypeptide, carbohydrate, lipid or combination thereof. It is  
25 typically a surface receptor which mediates endocytosis of a ligand. Thus, the binding agent can be a natural or synthetic ligand which binds the receptor. The ligand can be a protein, polypeptide, carbohydrate, lipid or a combination thereof which  
30 has functional groups that are exposed sufficiently to be recognized by the cell surface structure. It can also be a component of a biological organism such as a virus, cells (e.g., mammalian, bacterial, protozoan) or artificial carriers such as liposomes.

The binding agent can also be an antibody, or an analogue of an antibody such as a single chain antibody, which binds the cell surface structure.

Ligands useful in forming the carrier will vary according to the particular cell to be targeted. For targeting hepatocytes, glycoproteins having exposed terminal carbohydrate groups such as asialoglycoprotein (galactose-terminal) can be used, although other ligands such as polypeptide hormones may also be employed. Examples of asialoglycoproteins include asialoorosomucoid, asialofetuin and desialylated vesicular stomatitis virus. Such ligands can be formed by chemical or enzymatic desialylation of glycoproteins that possess terminal sialic acid and penultimate galactose residues. Alternatively, asialoglycoprotein ligands can be formed by coupling galactose terminal carbohydrates such as lactose or arabinogalactan to non-galactose bearing proteins by reductive lactosamination.

For targeting the molecular complex to other cell surface receptors, other types of ligands can be used, such as mannose for macrophages (lymphoma), mannose-6-phosphate glycoproteins for fibroblasts (fibrosarcoma), intrinsic factor-vitamin B12 for enterocytes and insulin for fat cells. Alternatively, the cell-specific binding agent can be a receptor or receptor-like molecule, such as an antibody which binds a ligand (e.g., antigen) on the cell surface. Such antibodies can be produced by standard procedures.



The poly- or oligonucleotide-binding agent complexes the oligonucleotide to be delivered. Complexation with the oligonucleotide must be sufficiently stable in vivo to prevent significant  
05 uncoupling of the oligonucleotide extracellularly prior to internalization by the target cell. However, the complex is cleavable under appropriate conditions within the cell so that the oligonucleotide is released in functional, hybridizable form.  
10 For example, the complex can be labile in the acidic and enzyme rich environment of lysosomes. A non-covalent bond based on electrostatic attraction between the binding agent and the oligonucleotide provides extracellular stability and is releasable  
15 under intracellular conditions.

Preferred poly- or oligonucleotide-binding agents are polycations that bind the negatively charged nucleic acid strands. These positively charged materials can bind noncovalently with the  
20 poly- or oligonucleotide to form a soluble, targetable molecular complex which is stable extracellularly but which releases the poly- or oligonucleotide as a functional (e.g., hybridizable) molecule intracellularly. Suitable polycations are  
25 polylysine, polyarginine, polyornithine, basic proteins such as histones, avidin, protamines and the like. A preferred polycation is polylysine (e.g., ranging from 3,800 to 60,000 daltons). Other noncovalent bonds that can be used to releasably link  
30 the poly- or oligonucleotide include hydrogen bonding, hydrophobic bonding, electrostatic bonding alone or in combination such as, anti-poly- or oligonucleotide antibodies bound to poly- or

oligonucleotide, and streptavidin or avidin binding to poly- or oligonucleotide containing biotinylated nucleotides.

The carrier can be formed by chemically linking  
05 the cell-specific binding agent and the oligonucleotide-binding agent. The linkage is typically covalent. A preferred linkage is a peptide bond. This can be formed with a water soluble carbodiimide as described by Jung, G. et al. (1981) Biochem.  
10 Biophys. Res. Commun. 101:599-606. An alternative linkage is a disulfide bond.

The linkage reaction can be optimized for the particular cell-specific binding agent and oligonucleotide-binding agent used to form the  
15 carrier. Reaction conditions can be designed to maximize linkage formation but to minimize the formation of aggregates of the carrier components. The optimal ratio of cell-specific binding agent to poly- or oligonucleotide-binding agent can be  
20 determined empirically. When polycations are used, the molar ratio of the components will vary with the size of the polycation and the size of the poly- or oligonucleotide. For example, for a conjugate of asialoorosomuroid linked to polylysine complexed with  
25 a 21-mer oligonucleotide the ratio can range from 2:1 to 1:2 by weight (asialoorosomuroid:oligonucleotide) can be used. Uncoupled components and aggregates can be separated from the carrier by molecular sieve or ion exchange chromatography. In a preferred  
30 embodiment, the conjugate is purified on a high pressure liquid cation exchange column (Aquapore™ cation-exchange, Rainin) with stepwise elution with 0.1 M sodium acetate, pH 5.0, 2.5, 2.25 and 2.0.

To form the complex, the poly- or oligonucleotide and carrier are mixed and incubated under conditions conducive to complexation. For example, the poly- or oligonucleotide and carrier can be mixed  
05 at the appropriate ratio in 2 M NaCl and the solution can be diluted to 0.15 M and filtered to provide an administrable composition.

The molecular complex can contain more than one copy of the nucleic acid strand. The amount of poly-  
10 or oligonucleotide, of course, should not exceed that required to maintain solubility of the resulting complex. The preferred weight or molar ratio of carrier to poly- or oligonucleotide for the construct employed can be determined by routine experimentation.

15 The molecular complex of this invention can be administered parenterally. Preferably, it is injected intravenously. The complex is administered in solution in a physiologically acceptable vehicle.

The molecular complex of this invention can be  
20 used to target delivery of a wide range of poly- and oligonucleotide for specific hybridization usually to an RNA target. The target can also be DNA by triplex formation. Duvall-Valentine et al. (1992) PNAS 89:504-508. In the former case, depending on the  
25 therapeutic goal, the oligonucleotide can be directed against translation of a gene or genes of cellular origin (e.g., a cellular oncogene) or of noncellular origin (e.g., a viral oncogene or the genes of an infecting pathogen such as a virus or a parasite such  
30 as malaria, trypanosome, lysteria or mycoplasma). In the latter cases, the antisense can be directed against transcription of target genes.

In one embodiment, the method of this invention can be used to treat hepatitis infection. The complex can be used to deliver an antisense oligodeoxynucleotide specifically to liver cells to block  
05 production of hepatitis virus. One strategy is to take advantage of the fact that, because of its compact nature, the human hepatitis B virus has only one polyadenylation signal (nucleotides 1903-1923). The signal is common to all hepatitis B viral-derived  
10 mRNA and is different from the mammalian signal. As a result, antisense oligodeoxynucleotides complementary to this region can block viral protein synthesis. In a preferred embodiment, the antisense strand is complexed to a carrier comprising a ligand  
15 for the hepatic asialoglycoprotein receptor and a polycationic protein such as polylysine to provide a soluble molecular complex targetable to the liver.

In another embodiment, the method of this invention can be used to alter the expression of a  
20 gene of cellular origin. This method may be useful in the treatment of diseases characterized by abnormal biosynthesis, especially overexpression, of normal or abnormal cellular proteins.

This invention is illustrated further by the following examples.

### Example 1

#### MATERIALS AND METHODS

##### 05 Cells and Cell Culture

Human hepatoma, HepG2 2.2.15 cells kindly provided by Dr. George Acs (Mt. Sinai School of Medicine, NY) and SK Hep1 cells were grown in DMEM and 10% fetal calf serum as described previously (Wu, 10 G.Y. and Wu, C.H. (1987) J. Biol. Chem. 262:4429-4432).

##### Preparation of Targetable Antisense DNA

A targetable, soluble DNA carrier was prepared by coupling asialoorosomucoid to poly L-lysine 15 (Sigma) ( $M_r = 59,000$ ) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce) as described previously (Wu, G.Y. and Wu, C.H. (1987) J. Biol. Chem. 262:4429-4432) except that the conjugate was purified by cation exchange chromatography using a 20 high pressure liquid chromatographic system (Rainin) employing an Aquapore C-300 column (Rainin) and stepwise elution with 0.1 M sodium acetate, pH 5.0, 2.5, 2.25 and 2.0. The second peak eluted from the column, as detected by U.V. absorption at 230 nm, was 25 determined to be the optimal conjugate form and was used for all subsequent experiments. A 21-mer oligodeoxynucleotide, complementary to a portion of the human hepatitis B virus (ayw subtype) (Hirschman, S.Z. et al. (1980) Proc. Natl. Acad. Sci. USA 30 77:5507-5511) including the polyadenylation signal,

corresponding to nucleotides 1903-1923 (See SEQUENCE ID NO. 1 - TTTATAAGGGTCGATGTCCAT) of the viral genome, was synthesized with phosphorothioate linkages on an automated nucleotide synthesizer (Applied Biosystems) (Matsukura, M. et al. (1987) Proc. Natl. Acad. Sci. USA 84:7705-7710). As a control, a random 21-mer sequence was prepared in an identical fashion. The purity of oligonucleotides was determined by electrophoresis through 15% polyacrylamide gels stained with ethidium bromide. Antisense DNA was titrated with conjugate to form a soluble complex using an agarose gel retardation system as described previously (Wu, G.Y. and Wu, C.H. (1987) J. Biol. Chem. 262:4429-4432) and a conjugate to DNA ratio of 1.6:1 by weight (asialoorosomucoid: DNA) was selected.

#### Assay for Receptor-Mediated Uptake of Complexed Antisense DNA

To evaluate uptake of oligonucleotides, antisense DNA was end-labeled with  $^{32}\text{P}$  (Sambrook, J. et al. (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, 2nd ed., vol. 2, pg. 11.31). DNA alone, or in the form of a complex was added to medium of HepG2 and SK Hep1 cells to make 50  $\mu\text{M}$  solutions with respect to added antisense DNA. Uptake was determined as described previously for asialoglycoproteins (Schwartz, A.L. et al. (1981) J. Biol. Chem. 256:8878-8881). In brief, cells were incubated with ligands at 37°C and at regular time intervals, dishes were chilled to 4°C, washed with cold 10 mM EDTA-phosphate buffered saline and the cell layers removed and scintillation

counted. To determine counts bound to the cell surface, identical sets of cells were incubated at 4°C with ligands and, after washing as described above, the cell layers were removed and adherent radioactivity was determined by scintillation counting. Uptake was calculated as the difference between total cell-associated counts at 37°C and counts bound to the cells at 4°C for each time point. All points were determined in triplicate and results shown as means  $\pm$  SEM expressed as pmole DNA/10<sup>6</sup> cells.

#### Antisense DNA and Viral Gene Expression

To determine the effect of antisense DNA on viral gene expression, HepG2 2.2.15 cells were seeded six days pre-confluence and incubated at 37°C in medium containing antisense DNA alone, complexed antisense DNA, complexed random DNA, or medium alone. All media containing added DNA were initially 50  $\mu$ M with respect to DNA. At daily intervals, 50  $\mu$ l of medium was sampled and assayed for hepatitis B surface antigen by an ELISA (Abbott) method as described by the manufacturer, modified for quantitation using serially diluted standard surface antigen (CalBiochem) which produced a linear response within the range of antigen levels found in the samples. Cell number was determined by microscopic counting cells stained with trypan blue. All points were determined in triplicate and the results of four experiments are shown as means  $\pm$  SEM expressed as  $\mu$ g/ml/10<sup>6</sup> cells.

Effect of Antisense DNA on Protein Secretion

HepG2 2.2.15 cells were incubated with 50  $\mu$ M complexed DNA as described in Figure 2 except that 10  $\mu$ Ci [ $^{35}$ S]-methionine (Amersham), specific activity 05 1000 Ci/mmol, was added to label newly synthesized proteins. After 24 hrs, medium was moved, cells washed with phosphate buffered saline and lysed with 1% sodium dodecyl sulfate which was subsequently removed with triton X-100. Both media and cell 10 lysates were treated with a specific rabbit anti-surface antigen antibody (DAKO) and precipitated with protein-A sepharose (Sigma). Precipitates were scintillation counted and each point assayed in triplicate. Total cell protein was determined by 15 colorimetric assay (Bio-Rad). The results of three experiments are shown as means  $\pm$  SEM expressed as cpm/mg cell protein.

In order to assess the effect of complexed antisense DNA on total protein synthesis, cells 20 treated with complexed DNA and labeled with [ $^{35}$ S]-methionine as described above were separated from media. Total protein was precipitated with 10% trichloroacetic acid and counted. The results of triplicate assays of three experiments are shown as 25 means  $\pm$  SEM expressed as cpm/mg cell protein.

Effect of Antisense DNA on Production of Viral DNA

Cells were incubated with 50  $\mu$ M antisense DNA alone, or in the form of complexes. After 24 hrs, medium was removed and HBV DNA, extracted from the 30 medium according to the method of Sells et al. (Sells, M.A. et al. (1987) Proc. Natl. Acad. Sci. USA 84:1005-1009) and from the cell layer according to



the method of Hirt (Hirt, B.J. (1967) Mol. Biol. 26:365-371). Total cell protein was determined by colorimetric assay (Bio-Rad). DNA extracted from equal volumes (40 ml) of medium and from approxi-  
05 mately equal numbers of cells ( $10^7$ ) were applied on an agarose gel. HBV DNA was identified by Southern blot using an EcoRI-BglIII fragment of the HBV genome (nucleotide 0 to 1982) as a probe labeled with  $^{32}\text{P}$  and exposed to x-ray film as described previously  
10 (Sambrook, J. et al. (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, 2nd ed., vol. 2, pp. 10.14-10.15). Relative quantitation was achieved by densitometry and confirmed by scintillation counting of corresponding bands.

15 The first objective was to determine whether a single-stranded DNA oligonucleotide could be bound by an asialoglycoprotein-based carrier and whether it could be specifically targeted to asialoglycoprotein receptor-bearing cells.

20 Figure 1 shows uptake of complexed antisense DNA by HepG2 and SK Hep1 cells. A targetable, soluble DNA carrier was prepared by coupling asialoorosomucoid to poly L-lysine using a water soluble carbodiimide as described previously (Wu, G.Y. and  
25 Wu, C.H. (1987) J. Biol. Chem. 262:4429-4432) and purified as described in Materials and Methods. The conjugate was complexed to a 21-mer oligodeoxynucleotide, complementary to a portion of the human hepatitis B virus including the polyadenylation  
30 signal and labeled with  $^{32}\text{P}$ . DNA alone, or in the form of a complex was added to medium to make it 50  $\mu\text{M}$  with respect to added antisense DNA. Uptake was determined as described previously for asialo-

glycoproteins (Schwartz, A.L. et al. (1981) J. Biol. Chem. 256:8878-8881). Cells were incubated at 37°C and at regular time intervals, dishes were chilled, washed with 10 mM EDTA-phosphate buffered saline. To  
05 determine counts bound to the cell surface, identical sets of cells were also incubated at 4°C with ligands and, after washing as described above, the cell layers were removed and radioactivity determined by scintillation counting. Uptake was calculated as the  
10 difference between cell-associated counts at 37°C and counts bound to the cell surface at 4°C for each time point. All points were determined in triplicate and results shown as means  $\pm$  SEM expressed as pmole DNA/ $10^6$  cells, (o), antisense DNA alone; ( $\blacklozenge$ ),  
15 complexed antisense DNA; ( $\Delta$ ), complexed antisense DNA plus a 100-fold molar excess of asialoorosomucoid. Figure 1 shows that in SK Hep1 [asialoglycoprotein receptor (-) cells], the average uptake of antisense DNA alone was less than 0.05 pmole/hr/ million cells  
20 over 4 hrs of exposure. Incubation with DNA in the form of a complex did not improve the uptake in these cells. Similarly, uptake of antisense DNA alone by HepG2 [asialoglycoprotein receptor (+)] cells was as low as observed in receptor (-) cells over the course  
25 of the 4 hrs. However, in the receptor (+) cells, complexed antisense DNA was taken up nearly linearly at an average rate 12 times faster than antisense DNA alone through the 4 hrs of incubation. Accumulation of complexed antisense was also 12 times greater than  
30 antisense DNA alone after 4 hrs of exposure. The uptake of complexed DNA was virtually completely blocked by administration of a large molar excess of free carrier protein, asialoorosomucoid, to compete

for receptor binding. This confirmed the involvement of asialoglycoprotein receptors in the differential delivery of the antisense DNA.

To determine whether the targeted antisense DNA was functional, effects on hepatitis B viral gene expression were evaluated. Figure 2 shows the effect of complexed antisense DNA on hepatitis B virus surface antigen concentration in culture medium. HepG2 2.2.15 cells were incubated at 37°C in medium containing antisense DNA alone, complexed antisense DNA, complexed random DNA, or medium alone. All media containing added DNA were initially 50 µM with respect to DNA. At daily intervals, medium was sampled and assayed for the presence of hepatitis B surface antigen by an ELISA (Abbott) method as described by the manufacturer, modified as described in Materials and Methods. Cell number was determined by microscopically counting cells stained with trypan blue. All points were determined in triplicate and the results of four experiments are shown as means  $\pm$  SEM expressed as µg/ml/10<sup>6</sup> cells.

Figure 2 shows that in untreated control cells, hepatitis B viral surface antigen steadily increased in concentration in their media, rising from 1 µg/ml/10<sup>6</sup> cells on the first day to 5.5 µg/ml/10<sup>6</sup> cells by the seventh day. Exposure of cells to antisense DNA alone had no significant effect until the 3rd day at which time surface antigen concentration was 30% lower than untreated controls. Nevertheless, surface antigen concentrations in the presence of antisense DNA alone continued to rise steadily throughout the 7 days of exposure. However, treatment with complexed antisense DNA resulted in an

80% inhibition after the 1st day and 95% inhibition by the 7th day compared to untreated controls. There was no significant increase in surface antigen concentration after the first 24 hrs. Complexed  
05 random DNA of the same size had no effect on antigen concentrations at any time point under identical conditions.

The lack of accumulation of hepatitis B surface antigen in the medium of cells treated with complexed  
10 antisense DNA could have been due to a block in protein secretion. To examine this possibility, the synthesis of new surface antigen was measured in the media and cell layers. Table 1A shows that radiolabeled immunoprecipitable surface antigen in  
15 both the medium and cells after treatment with complexed antisense DNA for 24 hrs were decreased to an equal extent (80%) compared to untreated cells. There was no significant intracellular accumulation of newly synthesized antigen that would have been  
20 expected if a block in protein secretion had occurred.

Table 1B shows that neither complexed antisense DNA nor random DNA had a significant effect on total newly synthesized protein in the cell layer. A small, 2%, decrease was detected in newly synthesized  
25 secreted protein in cells exposed to complexed antisense DNA. This likely reflects the contribution of the inhibition of viral surface antigen synthesis noted in Table 1A. The data, overall, indicate that the observed inhibition of hepatitis B surface  
30 antigen synthesis by complexed antisense DNA was specific and could not have been due to a generalized inhibition of total protein synthesis.

Table 1A  
Immunoprecipitable Hepatitis B Surface Antigen\*

<u>Treatment</u>	<u>Cell Layer+</u>	<u>Cell Medium+</u>
Untreated Control	56,100 $\pm$ 2,321	114,500 $\pm$ 2,442
05 Complexed Antisense DNA	10,200 $\pm$ 1,009	15,300 $\pm$ 890
Complexed Random DNA	52,500 $\pm$ 4,534	122,220 $\pm$ 5,742

Table 1B  
Total TCA Precipitable Radioactivity\*

<u>Treatment</u>	<u>Cell Layer+</u>	<u>Cell Medium+</u>
10 Untreated Control	184,498 $\pm$ 2,258	712,498 $\pm$ 5,435
Complexed Antisense DNA	188,844 $\pm$ 6,240	684,302 $\pm$ 9,678
Complexed Random DNA	183,591 $\pm$ 5,444	706,240 $\pm$ 7,544

\*after 24 hrs incubation  
+cpm/mg cell protein

15 TCA - trichloroacetic acid

Finally, in order to determine whether viral replication was affected, DNA was extracted from the medium and cells layers exposed to oligonucleotides for 24 hrs and analyzed by Southern blots. Figure 3 shows Southern blots of DNA extracted from medium and cells after 24 hrs exposure to antisense DNA. Cells were incubated as described for Figure 2 with 50  $\mu$ M antisense DNA alone, or in the form of complexes as described for Figure 2. After 24 hrs, medium was removed and DNA, extracted from the medium (Sells,

M.A. et al. (1987) Proc. Natl. Acad. Sci. USA 84:1005-1009) and from the cell layer (Hirt, B.J. (1967) Mol. Biol. 26:365-371). Total cell protein was determined by colorimetric assay (Bio-Rad). DNA  
05 extracted from equal volumes of medium and from approximately equal numbers of cells were applied on an agarose gel, HBV DNA was identified by Southern blot using an EcoRI-BglIII fragment of the HBV genome as a probe labeled with  $^{32}\text{P}$  and exposed to x-ray film  
10 (Sambrook, J. et al. (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, 2nd ed., vol. 2, pp. 10.14-10.15). Relative quantitation was achieved by densitometry and confirmed by scintillation counting of corresponding bands,  
15 normalized to equal volume or cell number, for media and cell layers, respectively. Duplicate blots were performed, a representative of which is shown above. Lanes 1-4, cell lysates; lanes 5-8, media; lanes 1 and 5, untreated controls; lanes 2 and 6, treated with  
20 complexed antisense DNA; lanes 3 and 7, treated with complexed random DNA and lanes 4 and 8, treated with antisense DNA alone. Expected positions for relaxed circular (RC) and single stranded (SS) forms are indicated on the right.

25        Figure 3 lanes 1 and 5 show that untreated cells produced bands at positions expected for relaxed circular and single-stranded linear viral replicative DNA forms. Other minor bands are present, at 2.3 kb for example, as described previously for this cell  
30 line (Sells, M.A. et al. (1987) Proc. Natl. Acad. Sci. USA 84:1005-1009). Lanes 2 and 6 show that treatment of cells with complexed antisense DNA decreased the amount of all viral DNA forms in the medium by

approximately 80% compared to untreated cells (lanes 1 and 5). Complexed random DNA, lanes 3 and 7, had no detectable effect on the levels of HBV DNA under identical conditions. Antisense DNA alone, lanes 4 and 8, decreased HBV DNA by approximately 30% relative to untreated controls. The number of viable cells as determined by trypan blue exclusion was not affected by treatment with any form of DNA (data not shown).

It has been shown previously that many cell-types are capable of taking up free oligonucleotides (Loke, S.L. et al. (1989) Proc. Natl. Acad. Sci. USA 86:3474-3478). Loke et al. showed that the rates of uptake were inversely proportional to the size of the oligonucleotide (Loke, S.L. et al. (1989) Proc. Natl. Acad. Sci. USA 86:3474-3478). However, in general, the longer the DNA sequence, the greater the specificity for target mRNA molecules. These conflicting properties illustrate two common problems with the current use of antisense oligonucleotides: inefficient uptake and lack of cell specificity. In order to improve uptake of antisense oligonucleotides, Lemaitre et al. covalently coupled an oligonucleotide to polylysine (Lemaitre, M. et al. (1987) Proc. Natl. Acad. Sci. USA 84:648-652) and obtained anti-viral effects at several-fold lower concentrations than could be obtained with free DNA. However, the delivery was not cell-specific. Our uptake data indicate that not only can transport of oligonucleotides into cells be greatly enhanced, but the uptake can also be directed to specific cells mediated by an asialoglycoprotein-based DNA-carrier system.

Because of the specificity of DNA hybridization with mRNA to form hybrids implicated in antisense-mediated inhibition of translation, antisense oligonucleotides have been used successfully to study normal gene expression in vitro (Bevilaqua, A. et al. (1988) Proc. Natl. Acad. Sci. USA 85:831-835). For similar reasons, antisense oligonucleotides have also been examined previously for anti-viral effects (Goodchild, J. et al. (1988) Proc. Natl. Acad. Sci. USA 85:5507-5511 and Agrawal, S. et al. (1989) Proc. Natl. Acad. Sci. USA 86:7790-7794). For example, Agrawal et al. administered infectious virus (HIV) together with antisense oligonucleotides in a non-targeted manner to cell media (Agrawal, S. et al. (1989) Proc. Natl. Acad. Sci. USA 86:7790-7794). Specific inhibition of viral replication was demonstrated. Similarly, Lemaitre et al. studied a model of acute viral infection in which antisense was pre-administered to cells (Lemaitre, M. et al. (1987) Proc. Natl. Acad. Sci. USA 84:648-652) with substantial specific antiviral effects. Our experiments differ from these previous studies in that our cells had a pre-existing, stable viral infection with viral production maintained by an integrated viral genome. Our data indicate that although a stable infection existed, delivery of antisense oligonucleotides can dramatically inhibit viral gene expression in a specific manner. However, in vivo, persistent production of hepatitis B virus is usually due to the presence of unintegrated viral DNA (Shafritz, D. et al. (1981) N. Eng. J. Med. 305:1067-1073). Integration of the viral genome into that of the host



is usually associated with a cessation of production of complete viral particles (Ganem, D. (1982) Rev. Infect. Dis. 4:1026). Whether targeted antisense delivery can be effective in the presence of an  
05 infection generated by unintegrated viral DNA remains to be seen. However, asialoglycoprotein uptake in hepatitis virus-infected HepG2 cells was found to be not substantially different from non-infected HepG2 cells (data not shown), indicating that infection by  
10 the virus did not alter the receptor activity in these cells. This suggests that targeted delivery of antisense oligonucleotides may be generally applicable to naturally infected hepatocytes, that are otherwise normal, via asialoglycoprotein receptors.

15 It should be noted that the oligonucleotides used in this current work were linked together by phosphorothioate bonds. These linkages are less susceptible to nuclease degradation than normal phosphodiester bonds. However, an antisense  
20 oligonucleotide synthesized with phosphodiester linkages was also effective. In addition, a variety of other synthetic strategies have been developed to confer nuclease resistance to antisense oligonucleotides (Miller, P.S. et al. (1985) Nucleosides  
25 Nucleotides 6:769-776). Forms that retain polyanionic character may also be deliverable by a receptor-mediated carrier system to provide enhanced and prolonged efficacy in a targeted manner.

Uptake of AsOR-Poly L-lysine-oligoDNA Complexes in Vivo

To determine whether the AsOR-poly-L-lysine-oligoDNA complexes retained their ability to be recognized by hepatocyte asialoglycoprotein receptors in vivo, rats 200-250 g, were injected intravenously via a tail vein with AsOR-poly-L-lysine-[<sup>32</sup>P]-oligoDNA alone or together with a 1000-fold molar excess of asialoorosomucoid in 0.15 M sterile saline. After 10 min, animals were killed and samples of blood, liver, spleen, lung and kidney were obtained. Tissue samples were weighed, homogenized, mixed with aqueous counting scintillant and counted for <sup>32</sup>P radioactivity on a beta-counter. The uptake of radioactivity by each organ was expressed as the mean of the percent of the total injected counts. These data indicate that complexed single-stranded (antisense) oligoDNA sequences can be targeted specifically to the liver by simple intravenous injection.

Organ Distribution of <sup>32</sup>P-(oligonucleotide) DNA  
as an AsOR-PL Complex

	<u>% injected</u>
Blood	6.0
Heart	2.1
Lung	4.5
25 Kidney	2.7
Spleen	3.7
Liver	81

## Competition with 1000-fold Molar Excess Cold AsOR

	<u>% injected</u>
Blood	74.1
Heart	1.5
05 Lung	3.2
Kidney	4.1
Spleen	3
Liver	14.1

Example 2

## 10 MATERIALS AND METHODS

ASSAYS FOR DETERMINING THE EFFECT OF AsOR-PL- $\alpha$ 1(I)  
PROCOLLAGEN ANTISENSE DNA ON PROCOLLAGEN BIOSYNTHESISCell Culture and Treatment

A mouse fibroblast cell line (3T3-AsGR) producing  
15 collagen and stably transfected with the  
asialoglycoprotein (AsG) receptor genes kindly  
provided by Dr. Michael Shia (Massachusetts Institute  
of Technology, Cambridge, MA) was grown in DMEM and  
10% FBS. Confluent 35mm dishes of 3T3-AsGR were  
20 treated with  $\alpha$ 1(I) antisense DNA alone (1.3  $\mu$ M or 2.7  
 $\mu$ M) or varying concentrations of antisense DNA complex  
(1.3  $\mu$ M, 1.7  $\mu$ M, or 2.7  $\mu$ M antisense) for 12-16 hours  
(overnight) at 37°C in DMEM and 10% FBS. The medium  
was removed and replaced with labeling medium  
25 containing 5  $\mu$ Ci/ml of [ $^3$ H]-proline, 50  $\mu$ g/ml  
L-ascorbate and varying concentrations of antisense  
DNA or complexes. Cells were incubated for

4 hours at 37°C in the labeling medium. Newly synthesized procollagens and other proteins were determined by bacterial collagenase digestion (Peterkofsky, B. and Diegelmann, Biochemistry 10:988-994 (1971)).

#### Collagenase Digestion

The labeling medium was removed and set aside. Buffer containing protease inhibitors (.5 M Tris, pH 7.4, .4 mM NEM, .2 mM PMSF, 2.5 mM EDTA) was added to the cell layer and the cell layer was removed and pooled with the supernatant. The entire mixture was homogenized using a Dounce homogenizer and ice cold TCA was added to a final concentration of 15%. TCA precipitable proteins were digested with bacterial collagenase (Form III, Advanced Biofactures, Lynbrook, NY) at 37°C for 2 hours followed by precipitation with TCA-tannic acid. The collagenase-sensitive radioactivity in the supernatant was separated by centrifugation to measure newly synthesized procollagen production. The collagenase-resistant precipitated radioactivity was used to calculate non-collagenous protein production. All assays were normalized to equal numbers of cells.

#### Preparation of Targetable Antisense DNA

A targetable, soluble DNA carrier was prepared by coupling asialoorosomucoid to poly L-lysine (Sigma) ( $M_r = 41,000$ ) using 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (Pierce) as described previously (Wu, G.Y. and Wu, C.H. (1987) J. Biol. Chem. 262:4429-4432) except that the conjugate was purified by cation exchange chromatography using a

high pressure liquid chromatographic system (Rainin) employing an Aquapore C-300 column (Rainin) and stepwise elution with 0.1 M sodium acetate, pH 5.0, 2.5, 2.25 and 2.0. The second peak eluted from the column, as detected by U.V. absorption at 230 nm, was determined to be the optimal conjugate form and was used for all subsequent experiments. A 24-mer oligodeoxynucleotide, complementary to the 5'-region of the pro  $\alpha 1$  procollagen mRNA (See SEQUENCE ID NO. 2 - CCGGAGGTCCACAAAGCTGAACAT) was synthesized with phosphodiester linkages on an automated nucleotide synthesizer (Applied Biosystems) (Matsukura, M. *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:7705-7710). Specifically, this sequence is antisense to the first 24 nucleotides of pro  $\alpha 1$  procollagen beginning at the  $\alpha 1(I)$  translation start site and including a portion of the first intron. The purity of oligonucleotides was determined by electrophoresis through 15% polyacrylamide gels stained with ethidium bromide. Antisense DNA was titrated with conjugate to form a soluble complex using an agarose gel retardation system as described previously (Wu, G.Y. and Wu, C.H. (1987) *J. Biol. Chem.* 262:4429-4432) and a conjugate to DNA ratio of 2:1 by weight (asialoorosomucoid-polylysine (AsOR-PL): DNA) was selected.

#### Assay for Receptor-Mediated Uptake of Complexed Antisense DNA

In order to characterize the uptake capacity of the asialoglycoprotein receptors on the 3T3-AsGR cells, confluent 35mm dishes of 3T3-AsGR cells in DMEM and 10% FBS were stripped with phosphate buffered

saline (PBS)-10 mM EDTA, pH 5.0 and incubated with 2  $\mu\text{g}/\text{ml}$  [ $^{125}\text{I}$ ]-AsOR with specific activity of  $1 \times 10^6$  cpm/ $\mu\text{g}$ . Uptake was determined as described previously for asialoglycoproteins (Schwartz, A.L. *et al.* (1981) *J. Biol. Chem.* 256:8878-8881). In brief, cells were incubated with [ $^{125}\text{I}$ ]-AsOR at 37°C and at regular time intervals (0.5, 1, 2, or 4 hours), dishes were chilled to 4°C, washed three times with cold 10 mM EDTA-phosphate buffered saline and the cell layers solubilized with 0.1 NaOH and gamma counted. This process was repeated by incubating the 3T3-AsGR cells with [ $^{32}\text{P}$ ]-DNA-AsOR-PL (1.3  $\mu\text{M}$  antisense) or 1.3  $\mu\text{M}$  DNA- $^{125}\text{I}$ -AsOR-PL (specific activity =  $0.5 \times 10^6$  cpm/ $\mu\text{g}$ ) to determine the rate of uptake of antisense complex except that cell layers incubated with  $^{32}\text{P}$ -labeled complex were scintillation counted. Parallel dishes were incubated with the radiolabeled ligand together with a 300-fold weight excess of cold AsOR to determine non-specific uptake. To ascertain counts bound to the cell surface, identical sets of cells were incubated at 4°C with ligands and, after washing as described above, the cell layers were removed and adherent radioactivity was determined by scintillation counting. Uptake was calculated as the difference between total cell-associated counts at 37°C and counts bound to the cells at 4°C for each time point.

#### RNA Extraction and RNA Dot Blots

Confluent 10 cm dishes of 3T3-AsGR cells in DMEM and 10% FBS were incubated overnight at 37°C with one or a combination of the following: (1)  $\alpha\text{l(I)}$  antisense oligonucleotide (1.3  $\mu\text{M}$  or 2.7  $\mu\text{M}$ ); (2) similar

concentrations of antisense complexed with AsOR-PL. Total cellular mRNA was extracted by the guanidinium thiocyanate method. (Chomczynski, P. and Sacchi, N., Anal. Biochem. 162: 156-159 (1987)). Quantity and  
05 quality of the extracted RNA were determined by  $A_{260}/A_{280}$  absorbance and ethidium bromide stained formaldehyde gels.

Dot blot studies starting with 20  $\mu$ g of total RNA were serially diluted 2-fold in 20x SSC pH 7.0.  
10 Samples were heat-denatured in 50% formamide, 7% formaldehyde, and 1x SSC pH 7.0 (Sambrook, J., et al. Molecular Cloning 7.54, 1989). Samples were applied to nitrocellulose filters using slot blot apparatus, cross-linked to the filter by ultraviolet exposure,  
15 and prehybridized for 3 hours at 42°C. The cDNA probes, labeled with  $^{32}$ P using a random priming method (Sambrook, J., et al. Molecular Cloning 13.44, (1989)) were rat pro $\alpha$ 1(I) and B-actin, both linearized with EcoRI, and a 900bp piece of rat pro $\alpha$ 2(I). The  
20 nitrocellulose filters were hybridized with the probes ( $0.5-1.0 \times 10^7$  cpm/filter) overnight at 42°C, washed, and exposed to film. Quantitation was done by scintillation counting of the filters.

#### Northern Blots

25 Total RNA samples (60  $\mu$ g) were heat denatured in 50% formamide, 2x formaldehyde running buffer, 7% formaldehyde at 55°C for 15 minutes (Sambrook, J., et al. Molecular Cloning 7.43, (1989)). Samples were run on formaldehyde gels (1% agarose, 2.2 M formaldehyde)  
30 for 4 hours at 100 mvolts on ice. Molecular weight

and control RNAs were stained in 0.1 ammonium acetate and 0.5 µg/ml ethidium bromide and photographed with a fluorescent ruler. The remaining gel was washed several times with water, the RNA hydrolyzed with 0.5 05 N NaOH for 20 minutes, and soaked in 20x SSC for 45 minutes. The RNA was transferred onto a nitrocellulose filter under vacuum for one hour and cross-linked to the filter by exposure to ultraviolet irradiation using a Stratalinker. Conditions for 10 prehybridization and hybridization were the same as in the slot blot studies.

## RESULTS

### Characterization of 3T3-AsGR cells

Since the 3T3-AsGR cells do not normally possess 15 asialoglycoprotein receptors, they were transfected with the asialoglycoprotein receptor genes and were found to have a  $K_d$  of  $1.5 \times 10^{-9}$  M, a binding saturation with [ $^{125}$ I]-AsOR at 1.8 µg/ml, and an uptake rate of [ $^{125}$ I]-AsOR of 1.8 pmole/million 20 cells/hr. The number of asialoglycoprotein receptors per cell was 250,000.

The 3T3-AsGR cells displayed a linear uptake of α1(I) antisense DNA-AsOR-PL complex up to 4 hours at a rate of 18.2 pmole of DNA/million cells/hr. In 25 contrast, the rate of uptake of labeled α1(I) antisense DNA alone was 0.15 pmole DNA/million cells/hr. Uptake of AsOR in a 300x excess by weight competed with the uptake of α1(I) antisense DNA complex.



Effect of  $\alpha 1(I)$  antisense DNA complex on collagen synthesis

$\alpha 1(I)$  antisense DNA complexes inhibited collagen production by 3T3-AsGR cells. This inhibition was  
 05 specific for collagen production and was dependent on the concentration of  $\alpha 1(I)$  antisense DNA in the complex. This result is set forth in the following table.

	$\mu M$	collagen	noncollagen	collagen
	$\alpha 1(I)$	proteins	proteins	production
		cpm/million cells		(% control)
	0	4,330	45,000	100
	1.3	3,190	44,500	74
	1.7	2,770	45,660	64
15	2.7	2,210	40,500	56

Effect of  $\alpha 1(I)$  antisense DNA complex on procollagen I mRNA levels

$\alpha 1(I)$  antisense DNA complex specifically inhibited mRNA of the  $\alpha 1(I)$  chain. But at a higher  
 20 concentration (2.7  $\mu M$ ) of antisense DNA complex, mRNA of both the  $\alpha 1(I)$  and  $\alpha 2(I)$  chain was inhibited. The following table is a result of the quantitation of several dot blot analyses.

	$\mu M$	$\alpha 1(I)$	$\alpha 2(I)$	$\beta$ -actin
		(% control)		
	0	100	100	100
	1.3	71	100	102
	2.7	65	85	106

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific  
05 procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Wu, George Y.

Wu, Catherine H.

05 (ii) TITLE OF INVENTION: Targeted Delivery of  
Poly- or  
Oligonucleotides to  
Cells

(iii) NUMBER OF SEQUENCES: 2

10 (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Lahive &amp; Cockfield

(B) STREET: 60 State Street

(C) CITY: Boston

(D) STATE: Massachusetts

15 (E) COUNTRY: U.S.A.

(F) ZIP: 02109

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

20 (C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: ASCII

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

25 (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/864,003

(B) FILING DATE: 03-APR-1992

(C) APPLICATION NUMBER: US 07/788,119

30 (D) FILING DATE: 04-NOV-1991

(E) APPLICATION NUMBER: US 07/755,083

(F) FILING DATE: 05-SEP-1991

-34-

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- 05 (A) LENGTH: 21 nucleotides  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (iv) ANTISENSE: yes
- 10 (ix) FEATURE: Complement to hepatitis B  
virus polyadenylation site
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTTATAAGGG TCGATGTCCA T

21

## 2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 24 nucleotides  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- 20 (iv) ANTISENSE: yes
- (ix) FEATURE: Complement to first 24 nucleotides of pro  $\alpha 1$   
procollagen beginning at the  $\alpha 1(I)$  translation  
start site and including a portion of the first  
intron.
- 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCGGAGGTCC ACAAAGCTGA ACAT

24

Claims

1. A soluble molecular complex for targeting a poly- or oligonucleotide to a specific cell for hybridization to an RNA therein, the complex comprising a single-stranded poly- or  
05 oligonucleotide which hybridizes to the RNA complexed with a carrier comprised of a cell-specific binding agent and a poly- or oligonucleotide-binding agent.
2. A soluble molecular complex of claim 1, wherein  
10 the oligonucleotide is an antisense oligodeoxynucleotide.
3. A soluble molecular complex of claim 2, wherein the antisense oligodeoxynucleotide hybridizes to cellular or viral RNA or DNA.
- 15 4. A soluble molecular complex of claim 3, wherein the antisense oligodeoxynucleotide hybridizes to a hepatitis viral RNA.
5. A soluble molecular complex of claim 4, wherein the antisense oligodeoxynucleotide hybridizes to  
20 the hepatitis RNA polyadenylation site.
6. A soluble molecular complex of claim 2, wherein the antisense oligodeoxynucleotide hybridizes to oncogene RNA.
7. A soluble molecular complex of claim 1, wherein  
25 the oligonucleotide is a ribozyme.

8. A soluble molecular complex of claim 1, wherein the oligonucleotide-binding agent is a polycation.
9. A soluble molecular complex of claim 8, wherein the polycation is polylysine.
10. A soluble molecular complex of claim 1, wherein the cell-specific binding agent binds a surface receptor of the cell which mediates endocytosis of the complex by the cell.
- 10 11. A soluble molecular complex of claim 10, wherein the cell-specific binding agent is a ligand for an asialoglycoprotein receptor and the targeted cell is an hepatocyte.
12. A soluble molecular complex of claim 11, wherein the ligand is an asialoglycoprotein.
13. A pharmaceutical composition, comprising a solution of the molecular complex of claim 1 and physiologically acceptable vehicle.
14. A soluble molecular complex for targeting an antisense oligodeoxynucleotide to an hepatocyte, the complex comprising the antisense oligodeoxynucleotide complexed with a carrier comprised of a ligand for the asialoglycoprotein receptor and an oligodeoxynucleotide-binding polycation.

15. A soluble molecular complex of claim 14, wherein the antisense oligodeoxynucleotide hybridizes to a viral RNA transcript.
- 05 16. A soluble molecular complex of claim 15, wherein the antisense oligodeoxynucleotide hybridizes to a hepatitis RNA transcript.
17. A soluble molecular complex of claim 16, wherein the antisense oligodeoxynucleotide hybridizes to a hepatitis virus RNA polyadenylation site.
- 10 18. A soluble molecular complex of claim 14, wherein the polycation is polylysine.
- 15 19. A soluble molecular complex, the complex comprising an antisense oligodeoxynucleotide complementary to hepatitis RNA polyadenylation site complexed with a carrier comprised of a ligand for the asialoglycoprotein receptor and an oligonucleotide-binding polycation.
20. A soluble molecular complex of claim 19, wherein the polycation is polylysine.
- 20 21. A method of delivering an oligonucleotide to a specific cell of an organism to block expression of a gene or genes in the cell, comprising administering to the organism a soluble molecular complex comprising a single-stranded oligonucleotide which hybridizes to an RNA transcript of the gene or genes, complexed with a carrier comprised of a cell-specific binding agent and an oligonucleotide-binding agent.
- 25

22. A method of claim 21, wherein the oligo-nucleotide is an antisense oligodeoxynucleotide.
23. A method of claim 22, wherein the oligodeoxy-nucleotide hybridizes to a viral RNA transcript.
- 05 24. A method of claim 23, wherein the oligodeoxy-nucleotide hybridizes to a hepatitis viral RNA transcript.
25. A method of claim 24, wherein the oligodeoxy-nucleotide hybridizes to a hepatitis viral RNA  
10 polyadenylation site.
26. A method of claim 21, wherein the oligo-nucleotide is a ribozyme which catalyzes the cleavage of the transcript.
27. A method of claim 21, wherein the oligonucleo-  
15 tide-binding agent is a polycation.
28. A method of claim 27, wherein the polycation is polylysine.
29. A method of claim 22, wherein the cell-specific  
20 binding agent binds a surface receptor of the cell which mediates endocytosis of the complex.
30. A method of claim 29, wherein the cell-specific binding agent is a ligand for an asialoglyco-protein receptor and the targeted cell is an hepatocyte.



31. A method of claim 30, wherein the ligand is an asialoglycoprotein.
32. A method of claim 21, wherein the molecular complex is administered intravenously.
- 05 33. A method of inhibiting replication of hepatitis virus in infected hepatocytes, comprising injecting a pharmaceutically acceptable solution of an effective amount of a molecular complex comprising an antisense oligodeoxynucleotide  
10 which hybridizes to an RNA transcript of a hepatitis DNA sequence necessary for viral replication, complexed with a carrier comprised of a ligand for the asialoglycoprotein receptor and an oligodeoxynucleotide-binding polycation.
- 15 34. A method of claim 33, wherein the oligodeoxynucleotide is complementary to a hepatitis viral RNA polyadenylation site.
35. A method of claim 33, wherein the polycation is polylysine.
- 20 36. A method of claim 33, wherein the molecular complex is administered intravenously.
37. An antisense oligonucleotide which hybridizes to the polyadenylation site of hepatitis virus.
- 25 38. An antisense oligodeoxynucleotide having the nucleotide sequence given as SEQUENCE ID NO. 1.

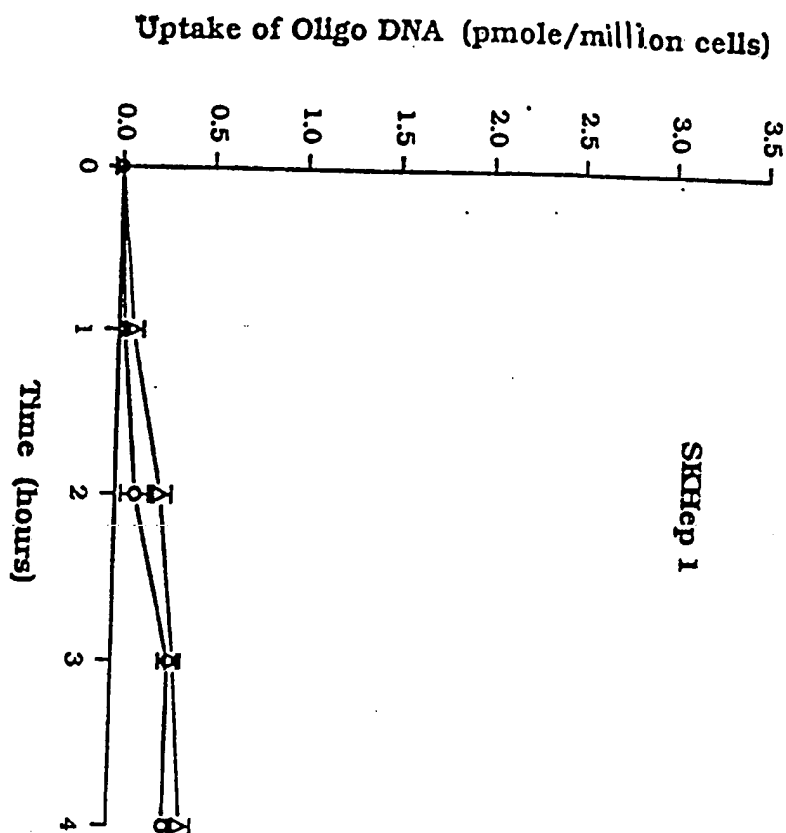


FIG. 1A

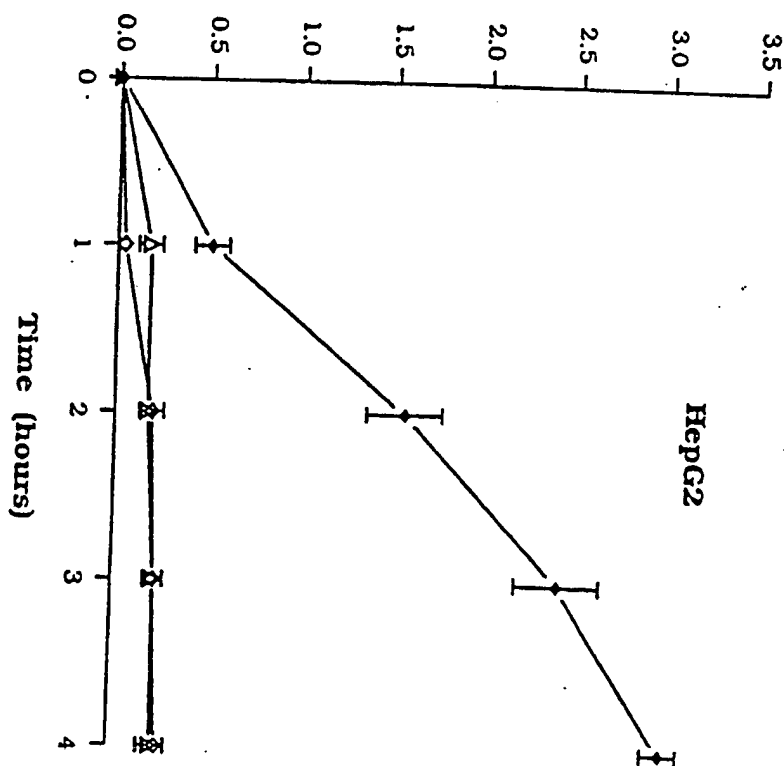


FIG. 1B

FIG. 2

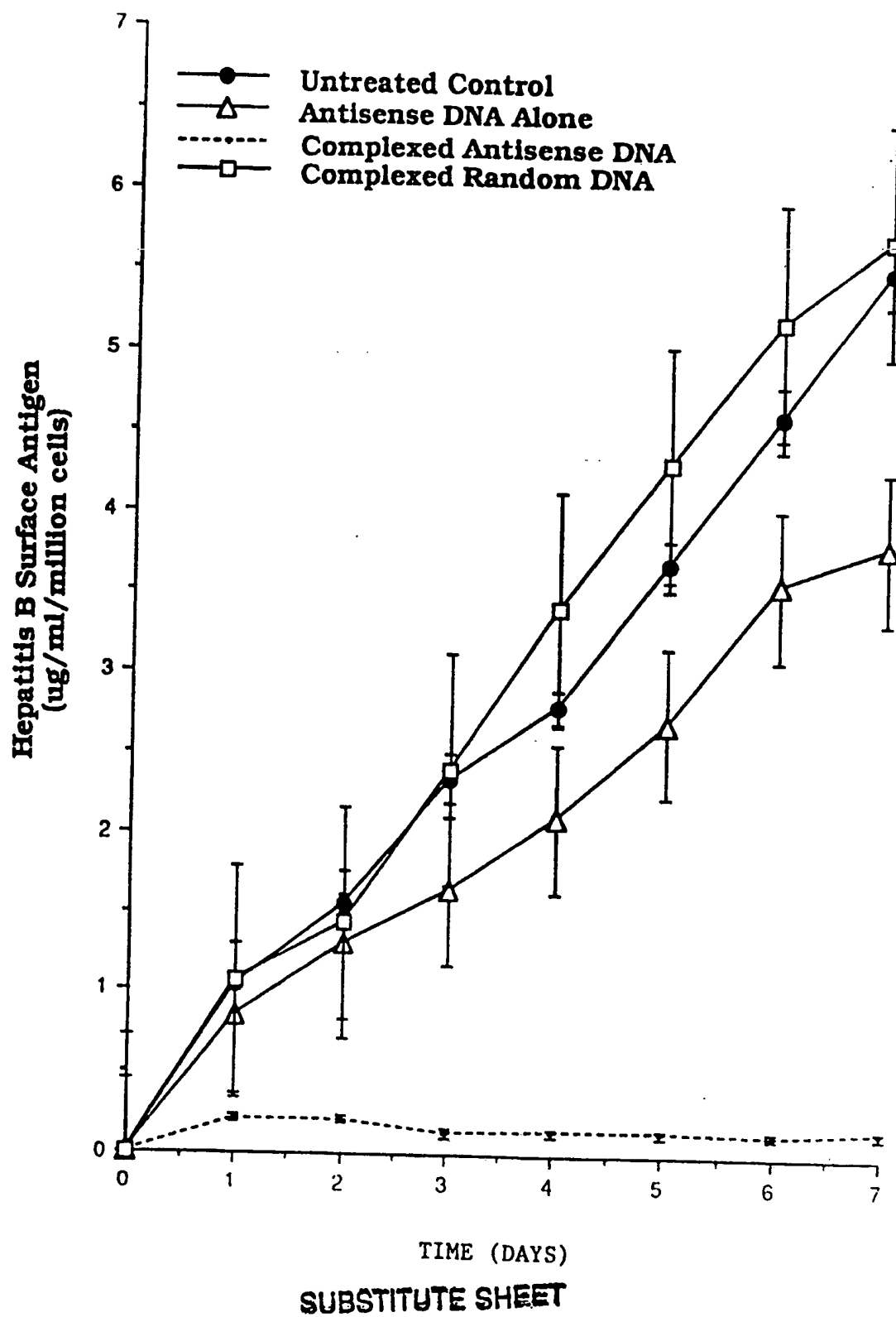
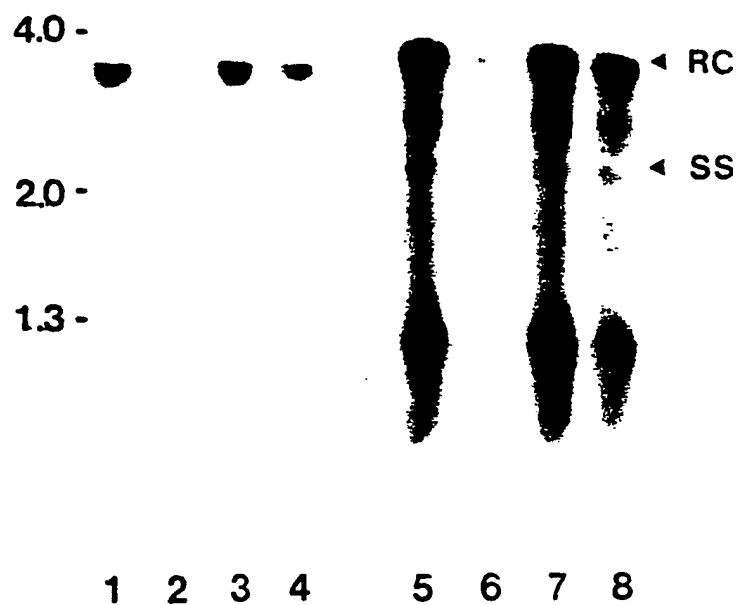


FIG. 3

kb



SUBSTITUTE SHEET

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/07339

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 48/00

US CL :514/44

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : NONE

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
noneElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
APS, Dialog/polycation, target (P) nucleotide!, antisense, receptor

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, volume 84, issued February 1987, Lemaitre <i>et al.</i> , "Specific antiviral activity of a poly (L-lysine)-conjugated oligodeoxyribonucleotide sequence complementary to vesicular stomatitis virus N protein mRNA initiation site", pages 648-652, see entire article.	1-3, 6-8, 10, 13, 15, 18, 19, 20, 22, 23, 27, 28
Y	Proceedings of the National Academy of Sciences, volume 77, no. 9, issued September 1980, Hirschman <i>et al.</i> , "Expression of cloned hepatitis B virus DNA in human cell cultures", pages 5507-5511, see entire article.	4, 5, 9, 16, 17, 24-26, 34, 37, 38
Y	Proceedings of the National Academy of Sciences, volume 87, issued May 1990, Wagner <i>et al.</i> , "Transferrin-polycation conjugates as carriers for DNA uptake into cells", pages 3410-3414, see entire article.	1-3, 6-8, 10, 13, 15, 18-20, 22, 23, 27, 28
Y	Journal of Biological Chemistry, volume 264, no. 29, issued 15 October 1989, Wu <i>et al.</i> , "Targeting genes: Delivery and persistent expression of a foreign gene driven by mammalian regulatory elements in vivo," pages 16985-16987, see entire article.	12, 14, 21, 29-33, 35, 36

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

13 NOVEMBER 1992

Date of mailing of the international search report

04 DEC 1992

Name and mailing address of the ISA/  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer

SUZANNE ZISKA

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196